

# Evidence for G-Protein Regulation of Inward $K^+$ Channel Current in Guard Cells of Fava Bean

Katrina Fairley-Grenot and Sarah M. Assmann<sup>1</sup>

The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138

Recent reports have shown that GTP-binding proteins (G-proteins) are present in plants but have given limited indication as to their site of action. G-proteins in animal cells transduce extracellular signals into intracellular or membrane-mediated events, including the regulation of ion channels. Using whole-cell patch clamp, we provide evidence that a G-protein in guard cells of fava bean regulates the magnitude (and not the kinetics) of inward current through  $K^+$ -selective ion channels in the plasma membrane.  $GDP\beta S$  (100 to 500  $\mu M$ ) increases inward  $K^+$  current, whereas  $GTP\gamma S$  (500  $\mu M$ ) has the opposite effect. The control nucleotides  $ADP\beta S$  and  $ATP\gamma S$  (500  $\mu M$ ) do not affect  $K^+$  current. Reduction of inward current by  $GTP\gamma S$  is eliminated in the presence of the  $Ca^{2+}$  chelator, BAPTA (1,2-bis(2-aminophenoxy)ethane- $N,N,N',N'$ -tetraacetic acid) (5 mM). When applied intracellularly, the G-protein regulators, cholera toxin and pertussis toxin, both decrease inward  $K^+$  current. The entry of  $K^+$  (and anions) into guard cells increases their turgor, opening stomatal pores in the leaf epidermis that allow gas exchange with the environment. Our data suggest the involvement of a G-protein in the inhibition of  $K^+$  uptake and stomatal opening. Changes in stomatal aperture, vital to both photosynthesis and plant water status, reflect guard-cell responsiveness to a variety of known environmental signals. The results presented here indicate that, in plants as well as animals, ion channel regulation by environmental stimuli may be mediated by G-proteins.

## INTRODUCTION

$K^+$  and anion fluxes across the plasma membrane of guard cells cause changes in stomatal aperture (Outlaw, 1983; Van Kirk and Raschke, 1978) and, therefore, are essential to the control of water status and carbon assimilation in plants. Stomatal opening results from an increase in intracellular osmotica due in large part to  $K^+$  influx through  $K^+$ -selective channels in the plasma membrane (see Zeiger, 1983; MacRobbie, 1987; Schroeder and Hedrich, 1989 for reviews). It has been postulated that channel-mediated  $K^+$  influx occurs as a response to a negative shift in membrane potential (hyperpolarization) caused by the activation of  $H^+$ -extruding ATPases (Assmann et al., 1985; Shimazaki et al., 1986) (see also Zeiger, 1983). Clint and Blatt (1989) suggest that energy-coupled  $K^+$  influx could also occur. The increase in intracellular osmotica increases guard cell turgor, widening the stomatal aperture due to mechanical constraints imposed by the radial micellation pattern of the guard cells (Aylor et al., 1973) and by the heavy lignification of the pore-side guard-cell wall (see Sharpe et al., 1987).

Stomatal opening occurs in response to a number of environmental stimuli, including light, low  $CO_2$  concentrations, and humidity (see Raschke, 1979; Zeiger, 1983 for reviews). There is a marked absence of information regarding the mechanisms by which these stimuli are trans-

duced into the ion fluxes that cause stomatal aperture change, although several lines of evidence indicate the involvement of  $Ca^{2+}$  in some transduction processes (see review by Mansfield et al., 1990).

In animal systems, regulation of ion channels by extracellular signals often involves GTP-binding proteins (G-proteins) (Brown and Birnbaumer, 1990). G-proteins have been found to couple membrane-bound receptors to ion channels both by cytoplasmically independent (membrane-delimited) pathways and by way of cytoplasmic intermediates such as  $Ca^{2+}$  (Brown and Birnbaumer, 1990). Evidence for G-protein regulation of ion channels depends on measurement of the effect on channel-mediated ion fluxes of molecules that perturb G-protein function. Such molecules include the nonhydrolysable GDP analog,  $GDP\beta S$ , that locks G-proteins in a GDP-bound, inactive form and the nonhydrolysable GTP analog,  $GTP\gamma S$ , that locks G-proteins in a GTP-bound, active form (Stryer and Bourne, 1986; Gilman, 1987). Furthermore, the diversity of G-protein effects in animal cells has led to their classification according to whether they are substrates for one, both, or neither of the ADP-ribosyltransferases, pertussis toxin (PTX) and cholera toxin (CTX) (Stryer and Bourne, 1986; Gilman, 1987). ADP-ribosylation by CTX typically activates G-proteins, while ADP-ribosylation of a different site by PTX typically results in inactivation.

<sup>1</sup> To whom correspondence should be addressed.

There is a steady accumulation of biochemical data showing that G-proteins also exist in plants. Most reports describe protein-binding assays using [ $^{35}\text{S}$ ]GTP $\gamma$ S. One such assay also uses antisera to the  $\alpha$ -subunit of animal G-proteins to identify G-proteins in the plasma membranes of fava beans, *Arabidopsis*, and *Commelina* (Blum et al., 1988). Some reports show that binding of G-proteins with [ $^{35}\text{S}$ ]GTP $\gamma$ S is affected by extracellular stimuli: blue light activates binding in pea (Warpeha et al., 1990), auxin enhances binding in rice (Zaina et al., 1990), and both red and far red light inhibit binding in *Lemna* (Hasunuma et al., 1987a). Hasunuma et al. (1987b) show that several G-protein fractions isolated from pea can be ADP-ribosylated by PTX. In addition, Hong et al. (1990) have recently cloned a G-protein  $\alpha$ -subunit gene from *Arabidopsis*.

There are two reports suggesting a role for G-proteins in plants. Bossen et al. (1990) show that the red light-induced swelling of mesophyll protoplasts from dark-grown wheat leaves is inhibited by GDP $\beta$ S. Their data suggest that the phosphoinositide pathway and the second messenger, cyclic-AMP, may be involved in regulating the ion fluxes that cause this swelling. Dillenschneider et al. (1986) found that guanine nucleotides stimulated the release of phosphoinositol derivatives from membranes isolated from *Acer*.

We report here evidence for G-protein regulation of ion channels in plants. We describe the effects of the G-protein regulators described above on  $\text{K}^+$  influx through channels in the plasma membrane of protoplasts isolated from guard cells of fava bean. Guard cells constitute a transduction system for which extracellular signals and ionic responses are already well documented (see Zeiger et al., 1987). Ion flux is measured by using the whole-cell patch clamp method (Hamill et al., 1981) because this allows perfusion of the cytoplasmic face of the plasma membrane with impermeant molecules known to regulate G-protein activity.

## RESULTS

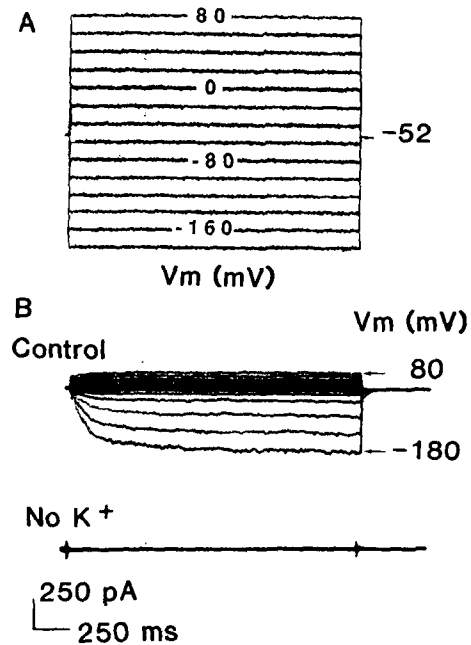
### Whole-Cell $\text{K}^+$ Current

A sequence of voltage pulses was applied to the membrane surrounding protoplasts from fava bean guard cells to measure the time and voltage dependence of whole-cell current under conditions where  $\text{K}^+$  was provided in the bath and pipette solutions as the chief permeant ion (see Methods). As shown in Figure 1A, the membrane potential ( $V_m$ ) was held at  $-52$  mV (at the calculated electrochemical equilibrium potential for  $\text{K}^+$ ,  $E_K$ ) and then stepped in 20 mV increments to a series of voltages ranging from  $-180$  to  $+80$  mV. There was a resting phase of 6 sec at the holding potential between each (2 sec) voltage pulse. Under control conditions, hyperpolarization of the membrane potential to potentials more negative than  $-100$  mV resulted in rapidly

activating inward current, indicating rapid activation (opening) of ion channels (Figure 1B, upper trace). (Inward current corresponds to the movement of positive charge ( $\text{K}^+$ ) into the pipette [i.e., into the protoplast from the external solution] and appears as downwardly directed time courses and negative current values.) Depolarization of the membrane potential to potentials more positive than  $-20$  mV resulted in the slower activation of a relatively small amount of outward current (Figure 1B, upper trace). There was no current when  $\text{K}^+$  was omitted from the bath and pipette solutions (Figure 1B, lower trace).

### Effect of Guanine Nucleotides

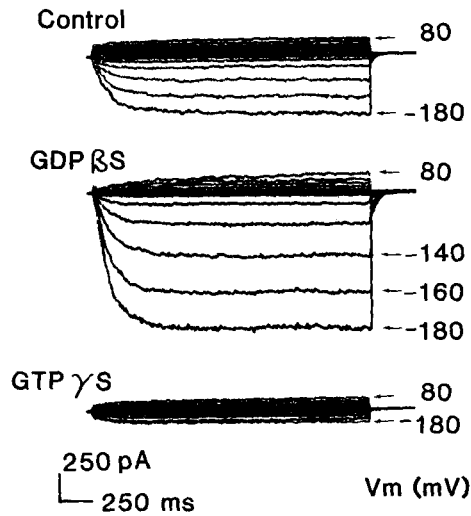
Figures 2 and 3 show that perfusion with GDP $\beta$ S of guard-cell protoplasts from fava bean resulted in an increase in the magnitude of time-activated inward current at a given (hyperpolarized) membrane potential. Differences are most obvious at extreme negative membrane potentials, where they are not obscured by the proximity of the membrane



**Figure 1.** Voltage Protocol and Whole-Cell Current in Guard Cell Protoplasts from Fava Bean.

(A) The upper trace shows the voltage ( $V_m$ ) protocol used to test the time and voltage dependence of whole-cell current in fava bean protoplasts.

(B) The lower traces show whole-cell current time courses measured using this protocol under control conditions (Control), and in the absence of  $\text{K}^+$  (No  $\text{K}^+$ ). Current records at the different membrane potentials have been superimposed.



**Figure 2.** Current Time Courses Measured in the Absence and Presence of Guanine Nucleotides.

Whole-cell current was measured in the absence of added nucleotide (Control, whole-cell seal resistance ( $R_{\text{seal}} = 1.5 \text{ G}\Omega$ ), or after perfusion of the cytoplasm with 500  $\mu\text{M}$  GDP $\beta\text{S}$  ( $R_{\text{seal}} = 1.6 \text{ G}\Omega$ ) or 500  $\mu\text{M}$  GTP $\gamma\text{S}$  ( $R_{\text{seal}} = 1.5 \text{ G}\Omega$ ). The similarity of seal resistances allows direct comparison of the three sets of traces as instantaneous current (see Methods) will be the same in all cases.

potential to  $E_K$  or by the intrinsic rectification of the channels. (Rectification refers to voltage regulation of the channels that results in a low probability of channel opening at membrane potentials between  $-80$  and  $-20 \text{ mV}$ .) The magnitude of the increase in inward current saturates as [GDP $\beta\text{S}$ ] increases (Figure 3). GDP $\beta\text{S}$  also resulted in activation of inward current at less negative membrane potentials ( $-80 \text{ mV}$  cf.  $-100 \text{ mV}$ ). Perfusion of the cytoplasm with the control nucleotide, ADP $\beta\text{S}$ , had no effect (Figure 3, open circles). ADP $\beta\text{S}$  is an important control both for nonspecific nucleotide effects and for addition of  $\text{Li}^+$ , because all nucleotides were applied as  $\text{Li}^+$  salts. Lithium is known to have limited permeability in these channels (Schroeder, 1988) and to be toxic to some plant cells (personal observation). Table 1A illustrates the statistical significance of the current-voltage data obtained during these treatments. Table 2 shows that the activation kinetics of the channels that conduct inward current did not change after GDP $\beta\text{S}$  (or ADP $\beta\text{S}$ ) treatment.

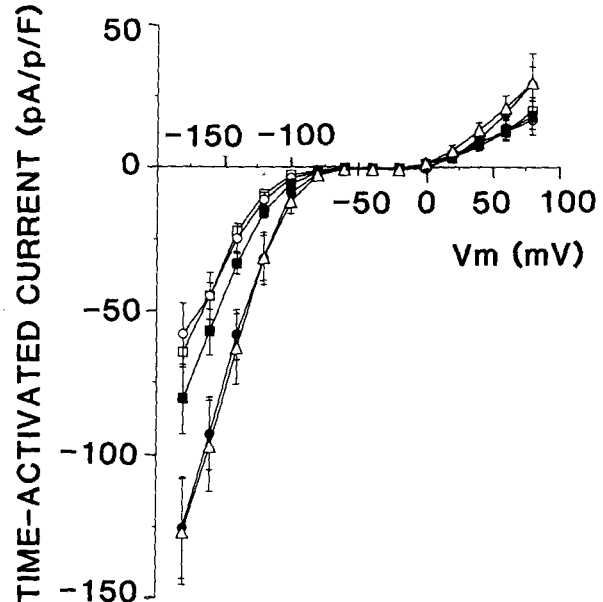
Figures 2 and 4 show that perfusion of protoplasts with GTP $\gamma\text{S}$  reduced the magnitude of time-activated inward K<sup>+</sup> current and shifted the activating voltage to more negative potentials ( $-120 \text{ mV}$  cf.  $-100 \text{ mV}$ ; Figure 4). It is important to note that the current scale in Figure 3 is double that used in Figure 4. This is a consequence of the opposite effects of GDP $\beta\text{S}$  (100% increase) and GTP $\gamma\text{S}$

(77% reduction) on inward current. ATP $\gamma\text{S}$  had no significant effect on K<sup>+</sup> current (Figure 4, open circles). Treatment with GTP $\gamma\text{S}$  did not affect current activation kinetics (Table 2). Results of statistical analysis of these data are given in Table 1B.

Neither the guanine nor the control (adenosine) nucleotides had any significant effect on outwardly directed K<sup>+</sup> current ( $P > 0.10$  for comparison of all treatments at  $V_m = +60, +80 \text{ mV}$ ).

### Is Ca<sup>2+</sup> Involved?

The effects of GTP $\gamma\text{S}$  on inward K<sup>+</sup> current in guard cells of fava bean are similar to the effects of increasing the cytoplasmic Ca<sup>2+</sup> concentration ( $\text{Ca}_i$ ). Schroeder and Hagiwara (1989) found that an increase in  $\text{Ca}_i$  from 0.1  $\mu\text{M}$  to 1.5  $\mu\text{M}$  inactivates inwardly rectifying K<sup>+</sup> channels in these protoplasts, shifting the membrane potential for channel activation from  $-87 \text{ mV}$  to  $-192 \text{ mV}$ . As shown in Figure 5 (closed squares), we also found that an increase in  $\text{Ca}_i$  (from 2 nM to 0.18  $\mu\text{M}$ ) decreased the amplitude of inward current and shifted the membrane potential at which current was detected to more negative values (cf. GTP $\gamma\text{S}$  treatment: Figure 5, closed triangles).



**Figure 3.** Effect of GDP $\beta\text{S}$  on Whole-Cell Current.

Time-activated, steady-state, whole-cell current is shown as a function of membrane potential ( $V_m$ ) after addition of 500  $\mu\text{M}$  ADP $\beta\text{S}$  ( $\circ$ ,  $n = 9$ ) or 0 ( $\square$ ,  $n = 7$ ), 50 ( $\blacksquare$ ,  $n = 4$ ), 100 ( $\bullet$ ,  $n = 4$ ), 500  $\mu\text{M}$  ( $\Delta$ ,  $n = 9$ ) GDP $\beta\text{S}$  to the pipette solution. Seal resistances (mean  $\pm$  SE) were (in  $\text{G}\Omega$ ):  $1.5 \pm 0.7$  ( $\circ$ ),  $1.5 \pm 0.4$  ( $\square$ ),  $1.5 \pm 0.8$  ( $\blacksquare$ ),  $2.5 \pm 0.9$  ( $\bullet$ ),  $3.0 \pm 0.6$  ( $\Delta$ ).

**Table 1.** Statistical Comparison of the Amplitude of Time-Activated Steady-State Current in the Presence and Absence of Guanine Nucleotides, Adenosine Nucleotides, and Toxins<sup>a</sup>

Treatments Compared	Range of $V_m$ at which Treatments Differed Significantly ( $P < 0.05$ )	Probability at $V_m = -180$ mV
<b>A</b>		
Control versus ADP $\beta$ S	None	$P < 0.68$
ADP $\beta$ S versus GDP $\beta$ S (50 $\mu$ M)	None	$P < 0.28$
ADP $\beta$ S versus GDP $\beta$ S (100 $\mu$ M)	$V_m \leq -100$ mV	$P < 0.0093$
ADP $\beta$ S versus GDP $\beta$ S (500 $\mu$ M)	$V_m \leq -120$ mV	$P < 0.0081$
<b>B</b>		
Control versus ATP $\gamma$ S	None	$P < 0.50$
ATP $\gamma$ S versus GTP $\gamma$ S	$V_m \leq -100$ mV	$P < 0.0000$
<b>C</b>		
Control versus BAPTA	None	$P < 0.67$
Control versus GTP $\gamma$ S/BAPTA	None	$P < 0.35$
BAPTA versus GTP $\gamma$ S/BAPTA	None	$P < 0.83$
<b>D</b>		
Control versus CTX <sub>i</sub>	$V_m \leq -120$ mV	$P < 0.0017$
Control versus PTX <sub>i</sub>	$V_m \leq -120$ mV	$P < 0.0016$

<sup>a</sup> Analyses of variance were performed on the current-voltage data obtained during different treatments. Analyses of variance were followed by two-tailed Student's *t* tests to assign probability (*P*) values to pairs of treatments. The number of cells in each treatment is the same as given in Table 2 and the figure legends.

To test whether GTP $\gamma$ S reduction of inward K<sup>+</sup> current requires cytoplasmic Ca<sup>2+</sup>, the effect of GTP $\gamma$ S was tested when a high concentration (5 mM) of the Ca<sup>2+</sup> chelator, BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid), was added to the standard pipette solution. The addition of BAPTA alone did not cause any significant change in the current amplitude (Table 1C) or kinetics (Table 2), suggesting that enhancement of inward K<sup>+</sup> current by lowering Ca<sub>i</sub> was already maximal at 2 nM Ca<sub>i</sub>. Figure 5 (open triangles) shows that in the presence of BAPTA, GTP $\gamma$ S had no significant effect: inward K<sup>+</sup> current was not reduced (Table 1C) and activation kinetics were not affected (Table 2).

### Effect of Toxins

Figures 6 and 7 show that in guard cells of fava bean, addition of either CTX or PTX (200 ng mL<sup>-1</sup>) to the cytoplasmic (pipette) solution inhibited inward K<sup>+</sup> current, a GTP $\gamma$ S-like response, without affecting channel kinetics (Table 2). Results of a statistical comparison of control and

toxin treatments are as given in Table 1D. Interestingly, when applied externally (incubation for 3 hr at 20°C in 500 ng mL<sup>-1</sup> toxin), PTX reduced inward K<sup>+</sup> current but CTX had no effect (data not shown).

### DISCUSSION

The voltage dependence, amplitude, kinetics, and K<sup>+</sup>-dependence of inward and outward current (Figure 1) indicate that they are conducted by previously characterized inwardly and outwardly rectifying K<sup>+</sup>-selective channel populations in the guard cell plasma membrane of fava beans (Schroeder et al., 1987; Schroeder, 1988).

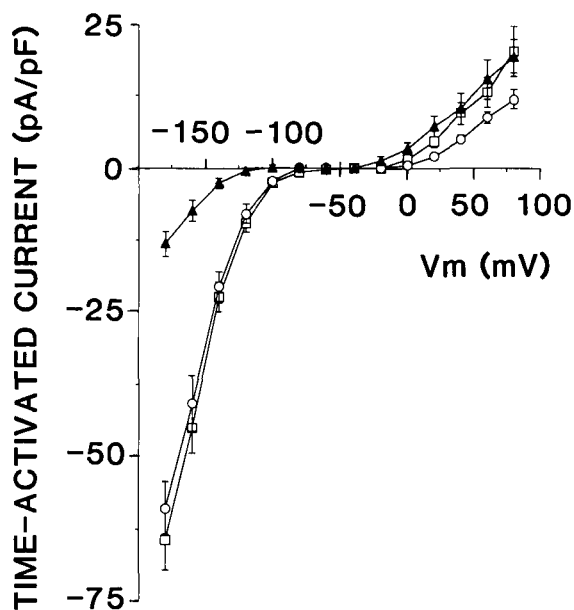
Our results are indicative of G-protein regulation of inwardly rectifying K<sup>+</sup> channels, with activation of the G-protein involved resulting in a decrease in inward K<sup>+</sup> current. The absence of an effect of the various treatments on the activation kinetics of inward current (Table 2) suggests that the G-protein does not regulate the rate of channel opening, but rather the (voltage-dependent) probability of channel opening or the single-channel conductance.

The results further suggest that the decrease in inward K<sup>+</sup> current that results from G-protein activation may result from an associated increase in Ca<sub>i</sub>. Ca<sub>i</sub> may increase by influx of Ca<sup>2+</sup> across the plasma membrane (Schroeder and Hagiwara, 1990; K. Fairley-Grenot and S.M. Assmann, unpublished results) and/or by release from intracellular stores (Hepler and Wayne, 1985; see also Schumaker and Sze, 1987; Gilroy et al., 1991). Recently, artificial elevation of cytosolic 1,4,5-trisphosphate (IP<sub>3</sub>) has been shown to increase Ca<sub>i</sub> in *Commelina* (Gilroy et al., 1990), which both

**Table 2.** Activation Half-Time for Time-Activated K<sup>+</sup> Current after Hyperpolarization to  $V_m = -180$  mV<sup>a</sup>

Treatment	Concentration	Half-time (msec)	<i>n</i>
Control	–	107 ± 8	7
ADP $\beta$ S	500 $\mu$ M	119 ± 25	9
GDP $\beta$ S	500 $\mu$ M	97 ± 14	9
ATP $\gamma$ S	500 $\mu$ M	86 ± 9	5
GTP $\gamma$ S	500 $\mu$ M	115 ± 11	8
GTP $\gamma$ S/BAPTA	500 $\mu$ M/5 mM	91 ± 26	4
BAPTA	5 mM	99 ± 18	5
PTX	200 ng/mL	129 ± 9	4
CTX	200 ng/mL	121 ± 17	5

<sup>a</sup> Half-time was measured by using a time- and current-labeled cursor to detect the point ( $\pm 10$  msec) in the current time course at which the time-activated current reached half of its steady-state amplitude. Values represent mean  $\pm$  SE for (*n*) protoplasts. Analysis of variance of the data used to construct this table shows that there is no significant difference between the activation half-times at  $V_m = -180$  mV of any of the treatments ( $P < 0.75$ ).



**Figure 4.** Effect of GTP $\gamma$ S on Whole-Cell Current.

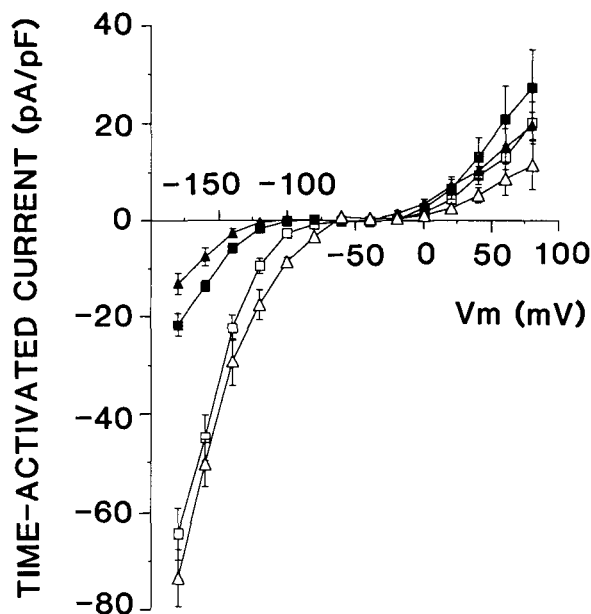
Time-activated, steady-state, whole-cell current as a function of membrane potential ( $V_m$ ) for protoplasts after addition of 500  $\mu$ M ATP $\gamma$ S ( $\circ$ ,  $n = 5$ ), 0 ( $\square$ ,  $n = 7$ ), or 500  $\mu$ M GTP $\gamma$ S ( $\blacktriangle$ ,  $n = 8$ ) to the pipette solution. Seal resistances (mean  $\pm$  SE) were (in G $\Omega$ ): 1.2  $\pm$  0.3 ( $\circ$ ), 1.5  $\pm$  0.4 ( $\square$ ), 1.0  $\pm$  0.3 ( $\blacktriangle$ ).

inhibits stomatal opening and triggers stomatal closure (Schwartz et al., 1988; Gilroy et al., 1990; McAinsh et al., 1990), and to decrease inward K<sup>+</sup> current (Blatt et al., 1990) in fava bean guard cells. IP<sub>3</sub> activates Ca<sup>2+</sup> efflux from the vacuole of red beet cells (Alexandre et al., 1990) and is known to liberate Ca<sup>2+</sup> from internal stores in animal cells (Berridge and Irvine, 1984). The G-protein regulation of both Ca<sub>i</sub> and IP<sub>3</sub> concentrations in several animal cell transduction systems is well documented (Petersen and Wakui, 1990).

G-proteins are known to regulate Ca<sup>2+</sup> channels and Ca<sup>2+</sup>-sensitive K<sup>+</sup> channels in the plasma membrane of a variety of animal cell types (Brown and Birnbaumer, 1990; Dolphin, 1991). Such regulation involves a variety of mechanisms (see Brown and Birnbaumer, 1990). Our results suggest at least three possible mechanisms for the observed inhibition of K<sup>+</sup> influx. G-protein activation may: (1) increase Ca<sub>i</sub> by causing Ca<sup>2+</sup> release from intracellular stores or by opening Ca<sup>2+</sup>-selective plasma membrane channels; (2) increase the Ca<sup>2+</sup> affinity of a regulatory Ca<sup>2+</sup> binding site on the inwardly rectifying K<sup>+</sup> channel protein; and (3) activate a Ca<sup>2+</sup>-independent pathway whose effects are detectable only at permissive Ca<sup>2+</sup> concentrations.

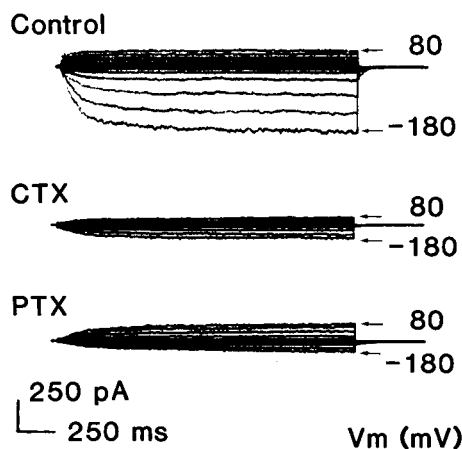
It may be possible to distinguish between (1) to (3) by testing the effects of G-protein regulators on single-channel activity in excised membrane patches. The success of such experiments will depend on what other (cytoplasmic) transduction components are required for G-protein regulation of K<sup>+</sup> influx.

In animal systems, a given G-protein may be activated by CTX and inhibited by PTX. Our toxin results suggest that the G-protein operating in the present plant system is one whose ADP-ribosylation by 200 ng mL<sup>-1</sup> of either CTX or PTX results in activation. Alternatively, the PTX effect may reflect inactivation of a second G-protein that, when activated, enhances inward K<sup>+</sup> current. Evidence for activation of such a G-protein may be provided by the data of Curvetto and Delmastro (1990), who observed a slight stimulation of stomatal opening in epidermal peels of fava bean incubated in the dark with high concentrations (10  $\mu$ g mL<sup>-1</sup>) of CTX. A comparable effect of CTX might have been absent from our protoplast system due to loss or



**Figure 5.** Comparison of the Effects of GTP $\gamma$ S and Internal [Ca<sup>2+</sup>] (Ca<sub>i</sub>) on Whole-Cell Current.

Time-activated, steady-state, whole-cell current is shown as a function of membrane potential ( $V_m$ ) for protoplasts after addition of 0 ( $\square$ ,  $n = 7$ ) or 500  $\mu$ M ( $\blacktriangle$ ,  $n = 8$ ) GTP $\gamma$ S or 500  $\mu$ M GTP $\gamma$ S plus 5 mM K<sub>4</sub>BAPTA ( $\triangle$ ,  $n = 4$ ) to the pipette solution. Addition of K<sub>4</sub>BAPTA increased [K<sup>+</sup>]<sub>i</sub> to 127 mM, shifting the K<sup>+</sup> equilibrium potential from -52 mV to -56 mV. Also shown is the current-voltage relationship in the absence of GTP $\gamma$ S but with higher Ca<sub>i</sub>: 0.18  $\mu$ M ( $\blacksquare$ ,  $n = 5$ ), cf. 2 nM ( $\square$ ). Seal resistances (mean  $\pm$  SE) were (in G $\Omega$ ): 1.5  $\pm$  0.4 ( $\square$ ), 1.0  $\pm$  0.3 ( $\blacktriangle$ ), 0.7  $\pm$  0.4 ( $\triangle$ ), 0.7  $\pm$  0.3 ( $\blacksquare$ ).



**Figure 6.** Current Time Courses Measured in the Absence and Presence of Toxins.

Whole-cell current was measured in the absence of toxin (Control, seal resistance ( $R_{\text{seal}} = 1.5 \text{ G}\Omega$ ) or after perfusion of the cytoplasm with 200 ng/mL CTX (CTX,  $R_{\text{seal}} = 2.9 \text{ G}\Omega$ ) or PTX (PTX,  $R_{\text{seal}} = 2.2 \text{ G}\Omega$ ).

alteration of membrane-situated glycolipids (Fishman, 1990) necessary for toxin binding and uptake; in contrast to the results of Figures 6 and 7, we observed no effect of CTX when applied externally.

In addition to inhibition of inward  $\text{K}^+$  current, both PTX and CTX caused a slight decrease in outward  $\text{K}^+$  current, although these results failed to achieve statistical significance, due to relatively large variability. Taken together, our data and those of others (Curvetto and Delmastro, 1990) may be suggestive of more than one G-protein operating in the regulation of stomatal aperture. Dual regulation of a pathway by opposing G-proteins is commonly observed in animal systems. The adenylate cyclase cascade provides one example of a major signal transduction system in animal cells that is regulated by two G-proteins, one which stimulates, and the other which inhibits, adenylate cyclase activity (see Stryer, 1988).

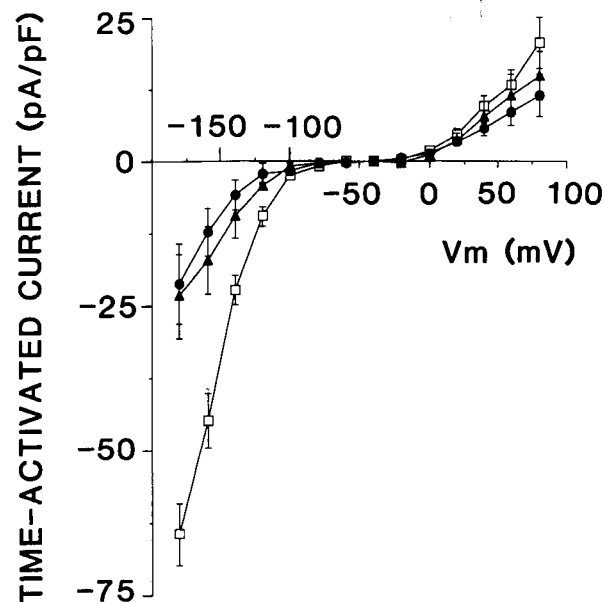
Our results also indicate that there may be a baseline level of G-protein activity under the experimental conditions used (i.e., in the absence of added stimulus; see Bourne et al., 1991) with this activity limiting the rate of  $\text{K}^+$  influx to a less-than-maximal value (control cf.  $\text{GDP}\beta\text{S}$  data, Figure 2). This complies with a working hypothesis that the G-protein regulation observed here is part of a mechanism by which one or more environmental signals are transduced into an inhibition of stomatal opening. The absence of red and blue light, which induce stomatal opening, and the external  $\text{Ca}^{2+}$  concentration (1 mM) are all experimental conditions that would inhibit stomatal opening (Assmann et al., 1985; Schwartz et al., 1988).

Work is under way to identify signals that may act through G-proteins in guard cells, and to link G-protein-mediated signal transduction with changes in stomatal aperture. Unfortunately, methods that would do this best (such as aperture measurements in epidermal peels) are subject to difficulties in internalizing G-protein regulators such as  $\text{GTP}\gamma\text{S}$  and  $\text{GDP}\beta\text{S}$ , a limitation bypassed by the cytoplasmic perfusion inherent to the whole-cell patch clamp method used in the present work.

## METHODS

### Protoplast Isolation

Guard cell protoplasts were isolated from the epidermis of the youngest expanded leaves of 3–4 week old plants of *Vicia faba* (cv Long Pod) according to established procedures (Kruse et al., 1989). Mean protoplast diameter was  $16.3 \pm 0.3 \mu\text{m}$  ( $n = 95$ ). After rinsing with 0.35 M mannitol plus 1 mM  $\text{CaCl}_2$ , protoplasts were kept in the dark on ice for at least 1 hr before being used in patch clamp experiments.



**Figure 7.** Effect of Toxins on Whole-Cell Current.

Time-activated, steady-state current as a function of membrane potential ( $V_m$ ) for protoplasts after addition of 0 ( $\square$ ,  $n = 7$ ) or 200 ng/mL cholera toxin ( $\blacktriangle$ ,  $n = 5$ ) or pertussis toxin ( $\bullet$ ,  $n = 4$ ) to the pipette solution. Seal resistances (mean  $\pm$  SE) were (in  $\text{G}\Omega$ ):  $1.5 \pm 0.4$  ( $\square$ ),  $4.4 \pm 1.1$  ( $\blacktriangle$ ),  $0.8 \pm 0.4$  ( $\bullet$ ).

### Patch Clamp Conditions

Protoplasts were placed in a bath solution containing 10 mM K<sup>+</sup> glutamate, 4 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM KOH, 10 mM Hepes, at pH 7.2 (KOH), adjusted to 450 mOsm kg<sup>-1</sup> with mannitol. The final [K<sup>+</sup>]<sub>o</sub> was 13.8 mM. Protoplasts were approached up to 4 hr later with patch pipettes pulled from Kimax-51 glass capillaries (VWR, Boston, MA) and filled with 98 mM K<sup>+</sup> glutamate, 2 mM MgCl<sub>2</sub>, 2 mM KCl, 10 mM Hepes, 2 mM EGTA, 2 mM MgATP (added daily) at pH 7.2 (KOH), adjusted to 500 mOsm kg<sup>-1</sup> with mannitol. The final [K<sup>+</sup>]<sub>i</sub> was 107 mM. In "No K<sup>+</sup>" experiments K<sup>+</sup> glutamate was replaced by *N*-methyl glucamine and glutamic acid. Free [Ca<sub>i</sub>] concentrations were calculated based on pH and concentrations of Ca<sup>2+</sup>, Mg<sup>2+</sup>, and EGTA. Liquid junction potentials were nulled. Test chemicals were added to the pipette solutions the day of application. Nucleotides were obtained as lithium salts from Calbiochem. BAPTA was obtained as the tetrapotassium salt from Sigma. Toxins were obtained from List Laboratories, Campbell, CA. PTX (6.06 μg mL<sup>-1</sup>) was activated before use by a 30-min incubation with 20 mM DTT at 35°C. PTX was subsequently added to pipette solution, resulting in final concentrations of 200 ng mL<sup>-1</sup> toxin and 0.66 mM DTT. Whole-cell clamping was performed at 20 ± 2°C under green light obtained by placing a Roscolene (Woburn, MA) No. 874 filter (peak transmitted wavelength: 520 nm, half-bandwidth 31 nm) in the light path.

### Current/Voltage Recording and Analysis

An Axopatch-1B (Axon Instruments, Burlingame, CA) amplifier connected to a microcomputer (Indec 11-73, Indec Inc., Sunnyvale, CA) by way of a multipurpose I/O device (Indec LDS) was used to voltage clamp protoplasts and to measure whole-cell (whole-protoplast) current. This is the current across the whole surface of a protoplast. Whole-cell current can only be measured after approaching a protoplast with a patch pipette, sucking a patch of membrane into the pipette tip and then rupturing that patch so that the protoplast interior is accessible to the pipette contents (see Hamill et al., 1981). After obtaining the whole-cell patch clamp configuration, the resting membrane potential and whole-cell capacitance were measured. Resting potentials varied between 0 and -56 mV and were typically about -15 mV. Whole-cell capacitance varied between 5 and 8 pF and was in most cases compensated by using the patch clamp amplifier. The membrane potential was then held at -52 mV before being stepped in 20-mV increments to voltages from -180 mV to +80 mV (as shown in Figure 1A). There was a resting phase of 6 sec at the holding potential between the (2 sec) voltage pulses. Whole-cell current was filtered at 2 kHz before being written to computer disk. Positive current is defined as the movement of positive charge out of the pipette.

Data reported in Figures 1 through 7 and in Tables 1 and 2 were collected by application of the pulse sequence 5 min after achieving the whole-cell configuration, to allow time for effective diffusional equilibration between the cytoplasm and the pipette solution, when calculated  $E_K = -52$  mV (the holding potential). Data are presented as current per unit whole-cell capacitance (pA pF<sup>-1</sup>) to normalize for variations in protoplast size. Data at longer times (up to 45 min) were collected when possible. These showed variations with time, but no consistent trends or, on average, significant differences from data at 5 min in any of the treatments.

Instantaneous current was measured immediately after decay of the capacitance transient, before activation of K<sup>+</sup> channels. This current is the sum of current through ion transport pathways open at the moment of the voltage change and current "leaking" through the membrane-pipette seal. Instantaneous current was linear with voltage under all experimental conditions and varied inversely with seal resistance. Steady-state current was measured as the mean value over a time period of 40 msec, beginning after 1.85 sec at the test potential. Time-activated current was calculated at a given membrane potential by subtracting the instantaneous current from the steady-state value. Values are reported as mean ± SE for (*n*) protoplasts. Analyses of variance were performed on the data to determine the significance of apparent differences in the current-voltage characteristic of the membrane under different treatments. Analyses of variance were followed by two-tailed Student's *t* tests to assign probability (*P*) values to pairs of treatments. These statistical analyses were performed on the same data as were used to construct Figures 3, 4, 5, and 7. Thus, these figures can be consulted to obtain means and SE of the treatments.

### ACKNOWLEDGMENTS

We thank J. Sherman for maintenance of fava bean plants and Dr. Susan F. Assmann for assistance with statistical analysis. This work was supported by National Science Foundation Grant DCB-8904041 and by a grant from the McKnight Foundation (S.M.A.) and by a Charles Gilbert Heydon Traveling Fellowship (K.F.-G.).

Received May 20, 1991; accepted July 29, 1991.

### REFERENCES

- Alexandre, J., Lassalles, J.P., and Kado, R.T. (1990). Opening of Ca<sup>2+</sup> channels in isolated red beet root vacuole membrane by inositol 1,4,5-trisphosphate. *Nature* **343**, 567-570.
- Assmann, S.M., Simoncini, L., and Schroeder, J.I. (1985). Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba*. *Nature* **318**, 285-287.
- Aylor, D.E., Parlange, J.Y., and Krikorian, A.D. (1973). Stomatal mechanics. *Am. J. Bot.* **60**, 163-171.
- Berridge, M.J., and Irvine, R.F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315-320.
- Blatt, M.R., Thiel, G., and Trentham, D.R. (1990). Reversible inactivation of K<sup>+</sup> channels of *Vicia* stomatal guard cells following the photolysis of caged inositol 1,4,5-trisphosphate. *Nature* **346**, 766-769.
- Blum, W., Hinsch, K.-D., Schultz, G., and Weiler, E.W. (1988). Identification of GTP-binding proteins in the plasma membrane of higher plants. *Biochem. Biophys. Res. Commun.* **156**, 954-959.
- Bossen, M.E., Kendrick, R.E., and Vredenberg, W.J. (1990). The involvement of a G-protein in phytochrome-regulated, Ca<sup>2+</sup>-dependent swelling of etiolated wheat protoplasts. *Physiol. Plant.* **80**, 55-62.

- Bourne, H.R., Sanders, D.A., and McCormick, F.** (1991). The GTPase superfamily: Conserved structure and molecular mechanism. *Nature* **349**, 117–127.
- Brown, A.M., and Birnbaumer, L.** (1990). Ionic channels and their regulation by G protein subunits. *Annu. Rev. Physiol.* **52**, 197–213.
- Clint, G.M., and Blatt, M.R.** (1989). Mechanisms of fusicoccin action: Evidence for concerted modulations of secondary K<sup>+</sup> transport in a higher plant cell. *Planta* **178**, 495–508.
- Curvetto, N., and Delmastro, S.** (1990). A biochemical and physiological proposal for stomatal movement: Possible involvement of adenosine 3',5'-cyclic monophosphate. *Plant Physiol. Biochem.* **28**, 367–378.
- Dillenschneider, M., Hetherington, A., Graziana, A., Alibert, G., Berta, P., Haiech, J., and Ranjeva, R.** (1986). The formation of inositol phosphate derivatives by isolated membranes from *Acer pseudoplatanus* is stimulated by guanine nucleotides. *FEBS Lett.* **208**, 413–417.
- Dolphin, A.C.** (1991). Regulation of calcium channel activity by GTP binding proteins and second messengers. *Biochim. Biophys. Acta* **91**, 68–80.
- Fishman, P.H.** (1990). Mechanism of action of cholera toxin. In *ADP-Ribosylating Toxins and G-Proteins*, J. Moss and M. Vaughan, eds (Washington, DC: American Society for Microbiology), pp. 127–140.
- Gilman, A.G.A.** (1987). G proteins: Transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**, 615–650.
- Gilroy, S., Read, N.D., and Trewavas, A.J.** (1990). Elevation of cytoplasmic calcium by caged calcium or caged inositol triphosphate initiates stomatal closure. *Nature* **346**, 769–771.
- Gilroy, S., Fricker, M.D., Read, N.D., and Trewavas, A.J.** (1991). Role of calcium in signal transduction of *Commelina* guard cells. *Plant Cell* **3**, 333–344.
- Hamill, O.P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F.J.** (1981). Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch. Eur. J. Physiol.* **391**, 85–100.
- Hasunuma, K., Furukawa, K., Funadera, K., Kubota, M., and Watanabe, M.** (1987a). Partial characterization and light-induced regulation of GTP-binding proteins in *Lemna paucicostata*. *Photochem. Photobiol.* **46**, 531–535.
- Hasunuma, K., Furukawa, K., Tomita, K., Mukai, C., and Nakamura, T.** (1987b). GTP-binding proteins in etiolated epicotyls of *Pisum sativum* (Alaska) seedlings. *Biochem. Biophys. Res. Commun.* **148**, 133–139.
- Hepler, P.K., and Wayne, R.** (1985). Calcium and plant development. *Annu. Rev. Plant Physiol.* **36**, 397–439.
- Hong, M., Yanofsky, M.F., and Meyerowitz, E.M.** (1990). Molecular cloning and characterization of GPA1, a G protein subunit gene from *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **87**, 3821–3825.
- Kruse, T., Tallman, G., and Zeiger, E.** (1989). Isolation of guard cell protoplasts from mechanically prepared epidermes of *Vicia faba* leaves. *Plant Physiol.* **90**, 1382–1386.
- MacRobbie, E.A.C.** (1987). Ionic relations in guard cells. In *Stomatal Function*, E. Zeiger, G.D. Farquhar, and I.R. Cowan, eds (Stanford, CA: Stanford University Press), pp. 125–162.
- Mansfield, T.A., Hetherington, A.M., and Atkinson, C.J.** (1990). Some current aspects of stomatal physiology. *Annu. Rev. Plant Physiol.* **41**, 55–75.
- McAinsh, M.R., Brownlee, C., and Hetherington, A.M.** (1990). Abscisic acid-induced elevation of guard cell cytosolic Ca<sup>2+</sup> precedes stomatal closure. *Nature* **343**, 186–188.
- Outlaw, W.H.** (1983). Current concepts on the role of potassium in stomatal movements. *Physiol. Plant.* **59**, 302–311.
- Petersen, O.H., and Wakui, M.J.** (1990). Oscillating Ca<sup>2+</sup> signals evoked by activation of receptors linked to inositol lipid hydrolysis: Mechanism of generation. *Membr. Biol.* **118**, 93–102.
- Raschke, K.** (1979). Movements of stomata. In *Encyclopedia of Plant Physiology*, New Series, Vol. 7, W. Haupt, and E. Feinleib, eds (Berlin: Springer-Verlag), pp. 383–441.
- Schroeder, J.I.** (1988). K<sup>+</sup> transport properties of K<sup>+</sup> channels in the plasma membrane of *Vicia faba* guard cells. *J. Gen. Physiol.* **92**, 667–683.
- Schroeder, J.I., and Hagiwara, S.** (1989). Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature* **338**, 427–430.
- Schroeder, J.I., and Hagiwara, S.** (1990). Repetitive increases in cytosolic Ca<sup>2+</sup> of guard cells by abscisic acid activation of nonselective Ca<sup>2+</sup> permeable channels. *Proc. Natl. Acad. Sci. USA* **87**, 9305–9309.
- Schroeder, J.I., and Hedrich, R.** (1989). Involvement of ion channels and active transport in osmoregulation and signaling in higher plants. *Trends Biochem. Sci.* **14**, 187–192.
- Schroeder, J.I., Raschke, K., and Neher, E.** (1987). Voltage dependence of K<sup>+</sup> channels in guard cell protoplasts. *Proc. Natl. Acad. Sci. USA* **84**, 4108–4112.
- Schumaker, K.S., and Sze, H.** (1987). Inositol-1,4,5-trisphosphate releases Ca<sup>2+</sup> from vacuolar membrane vesicles of oat roots. *J. Biol. Chem.* **262**, 3994–3946.
- Schwartz, A., Ilan, N., and Grantz, D.A.** (1988). Calcium effects on stomatal movement in *Commelina communis* L. *Plant Physiol.* **87**, 583–587.
- Sharpe, P.J.H., Wu, H., and Spence, R.D.** (1987). Stomatal mechanics. In *Stomatal Function*, E. Zeiger, G.D. Farquhar, and I.R. Cowan, eds (Stanford, CA: Stanford University Press), pp. 91–114.
- Shimazaki, K., Iino, M., and Zeiger, E.** (1986). Blue light-dependent proton extrusion by guard cell protoplasts of *Vicia faba*. *Nature* **319**, 324–326.
- Stryer, L.** (1988). *Biochemistry*, 3d ed (New York: Freeman).
- Stryer, L., and Bourne, H.R.A.** (1986). G proteins: A family of signal transducers. *Rev. Cell Biol.* **2**, 391–419.
- Van Kirk, C.A., and Raschke, K.** (1978). Release of malate from epidermal strips during stomatal closure. *Plant Physiol.* **61**, 474–475.
- Warpeha, K.M.F., Hamm, H.E., and Kaufman, L.S.** (1990). A blue-light-induced plant-membrane-associated GTP-binding protein in pea. *Plant Physiol.* **93S**, 30.
- Zaina, S., Reggiani, R., and Bertani, A.** (1990). Preliminary evidence for involvement of GTP-binding protein(s) in auxin signal transduction in rice (*Oryza sativa* L.) coleoptile. *J. Plant Physiol.* **136**, 653–658.
- Zeiger, E.** (1983). The biology of stomatal guard cells. *Annu. Rev. Plant Physiol.* **34**, 441–475.
- Zeiger, E., Farquhar, G.D., and Cowan, I.R.** (1987). *Stomatal Function*, (Stanford, CA: Stanford University Press).



**Evidence for G-Protein Regulation of Inward K<sup>+</sup> Channel Current in Guard Cells of Fava Bean.**

K. Fairley-Grenot and S. M. Assmann

*Plant Cell* 1991;3;1037-1044

DOI 10.1105/tpc.3.9.1037

This information is current as of October 29, 2020

<b>Permissions</b>	<a href="https://www.copyright.com/ccc/openurl.do?sid=pd_hw1532298X&amp;iissn=1532298X&amp;WT.mc_id=pd_hw1532298X">https://www.copyright.com/ccc/openurl.do?sid=pd_hw1532298X&amp;iissn=1532298X&amp;WT.mc_id=pd_hw1532298X</a>
<b>eTOCs</b>	Sign up for eTOCs at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a>
<b>CiteTrack Alerts</b>	Sign up for CiteTrack Alerts at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a>
<b>Subscription Information</b>	Subscription Information for <i>The Plant Cell</i> and <i>Plant Physiology</i> is available at: <a href="http://www.aspb.org/publications/subscriptions.cfm">http://www.aspb.org/publications/subscriptions.cfm</a>